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Endobronchial Photodynamic Therapy: Comparison of mTHPC and Polyethylene Glycol-Derived mTHPC on Human Tumor Xenografts and Tumor-Free Bronchi of Minipigs

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Background and Objective: **Photodynamic therapy (PDT) with mTHPC and polyethylene glycol-derived mTHPC (pegylated mTHPC) was compared on nude mice bearing human squamous cell carcinoma and adenocarcinoma xenografts. The same treatment regimens were applied to the bronchi of tumor-free minipigs to assess injury to normal tissue.**
Study Design/Materials and Methods: **Laser light (652 nm, 20 J/cm^2) was delivered as surface radiation to the xenografts 4 days after intraperitoneal administration of 0.1 mg/kg mTHPC or an equimolar dose of pegylated mTHPC, respectively. The extent of tumor necrosis was assessed by histomorphometry. Endobronchial PDT was performed on the bronchi of minipigs with the same drug and light doses at drug-light intervals ranging from 12–96 hr.**
Results: **Both sensitizers produced larger necrosis of squamous cell carcinoma and adenocarcinoma xenografts than was observed in untreated controls ($P < 0.005$). Pegylated mTHPC led to larger tumor necrosis than mTHPC in squamous cell carcinoma ($P < 0.001$), but not in adenocarcinoma xenografts. mTHPC-PDT resulted in ulceration and necrosis of bronchial mucosa in minipigs at drug-light intervals ranging from 12–48 hr, which was not observed after use of pegylated mTHPC.**
Conclusions: **In this setting, pegylated mTHPC had advantages as a photosensitiser compared to mTHPC. Lasers Surg. Med. 23:25–32, 1998.** **© 1998 Wiley-Liss, Inc.**

Key words: adenocarcinoma; m-tetrahydroxyphenylchlorin; nude mice; squamous cell carcinoma

INTRODUCTION

Photodynamic therapy (PDT) is an attractive antitumor treatment since it can destroy superficially localized tumors while sparing underlying tumor-free structures. Potential indications for

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endobronchial application include: (1) small carcinomas in patients who are not candidates for major resections, (2) carinal tumors in order to avoid sleeve pneumonectomy or carinal resection, (3) remaining tumor after incomplete resections, or (4) stump recurrences after resection, and (5) to prolong palliation after stenting or tumor ablation by use of Nd:YAG laser or brachytherapy [1]. An especially promising application of PDT seems to be in situ carcinoma of the pharynx, esophagus, and tracheo-bronchial tree [2]. A phase II study has shown an excellent effect of PDT on patients with centrally located, early-stage lung cancer with limited tumor invasion extending over a small area [3].

PDT, however, may cause injury to normal structures, especially if Photofrin, a first-generation sensitiser, is given [4–10]. To overcome the drawbacks of Photofrin, new sensitisers are currently being developed [11]. We have tested m-tetrahydroxyphenylchlorin (mTHPC), a chlorin class compound, in clinical and experimental settings. In patients suffering from malignant mesothelioma, 0.3 mg mTHPC/kg and 10 J/cm^2 resulted in tumor necrosis up to 10 mm depth, but significant side effects were observed with this treatment regimen [12]. In nude mice bearing human mesothelioma xenografts, the tumor selectivity was enhanced by increasing the drug-light interval up to 4 days [13] and decreasing the drug dose to 0.1 mg/kg while increasing the light dose to 20 J/cm^2 [14].

This study focuses on the potential for endobronchial application of mTHPC-PDT and on the possibility to increase tumor selectivity by structural modifications of the photosensitizer mTHPC by covalently binding polyethylene glycol to mTHPC (pegylated mTHPC). Polyethylene glycol is a long chain, water-soluble polyether that is widely used as a pharmaceutical vehicle. Recently it has been found that covalent binding of polyethylene glycol to pharmaceuticals confers additional benefits beyond those seen when used as a vehicle alone [15,16]. These include decreased immunogenicity and liver absorption, increased drug circulation time, and enhanced tumor targeting for anticancer drugs. The photosensitizing effect of mTHPC was compared to that of pegylated mTHPC on nude mice with subcutaneously implanted human squamous cell carcinoma or adenocarcinoma xenografts and on tumor-free bronchi of minipigs, using the same drug-light conditions, in order to assess for both sensitizers the tumoricidal effect on xenografts in mice and the alterations of normal bronchi in minipigs.

MATERIALS AND METHODS

Animals and Housing

Poorly differentiated human squamous cell carcinoma [15] and poorly differentiated human adenocarcinoma [17] were xenografted subcutaneously onto BALB/c nude mice using the trocar technique. At least six passages on nude mice were performed for each tumor type before treatment was initiated. The tumor volume doubling time was 113 hr for squamous cell carcinoma and 135 hr for adenocarcinoma xenografts. One tumor was grown subcutaneously on the right flank of each animal and treatment was initiated when the tumor reached a diameter of 10–12 mm. Animal housing included artificial light in a 12-hr rhythm before and after administration of the photosensitiser.

Minipigs with a body weight of 23–29 kg were housed in facilities of the University Animal Hospital (Bern, Switzerland). They were cared for by professional veterinary staff (DM) and were examined daily after endobronchial light delivery in order to assess photosensitising effects of each drug. All animals were treated according to the European Convention on Animal Care. The study was approved by the Ethics Committee of the University of Bern.

Drug Administration

m-tetrahydroxyphenylchlorin (mTHPC) [18] from Scotia Pharmaceuticals (Guildford, UK) was dissolved in a pharmaceutical-grade solution of 20% ethanol, 30% polyethylene glycol 400 and 50% H_2O for administration. Polyethylene glycol-derived mTHPC (pegylated mTHPC) from Scotia is a water-soluble, high molecular weight derivative of mTHPC, prepared by covalently binding polyethylene glycol 5,000, to each of the four hydroxy residuals to give tetrakis (methoxy PEG 5,000) ether of 7,8 dihydro-5,10,15,20-tetrakis-(3-hydroxyphenyl)-21, 23-(H)-porphyrin. Its molecular weight is ~20,000. It was dissolved in sterile 0.9% NaCl for administration.

A dose of 0.1 mg/kg mTHPC, and an equimolar dose of pegylated mTHPC were administered such that the amount of mTHPC (the active moiety) used was the same in both groups, regardless of the molecular weight of the compound. Twelve mice received mTHPC and 12 mice

received pegylated mTHPC, by i.p. injection; five minipigs received mTHPC and five minipigs received pegylated mTHPC by i.v. administration.

Light Delivery

Mice bearing xenografts. Two groups of six animals each, bearing squamous cell carcinoma and adenocarcinoma, respectively, were treated with laser light 4 days after mTHPC administration. Two groups of six animals each, bearing squamous cell carcinoma and adenocarcinoma, respectively, received laser light 4 days after injection of pegylated mTHPC. The tumors were 10–12 mm in diameter at initiation of treatment. Before light delivery, the animals were anaesthetized with Avertin i.p. (Sterling Winthrop, New York) and kept on a warm towel pad during treatment. Argon-pumped dye laser light of 652 nm was delivered through a quartz optical fiber containing a lens. A power track system allowed for a constant power output (Coherent Innova 200 and Dye CR 599, GMP SA, Lausanne, Switzerland). In each animal, noncontact surface irradiation was performed on the tumor through the intact skin overlying the tumor and on an equally sized area of the hind leg serving as control site. The irradiated spots were 1.3 cm in diameter and the treated surfaces were situated perpendicular to the incident laser beam. The power at the end of the fiber was measured by a power meter calibrated for 652 nm, allowing for nonthermal power density on the irradiated surfaces of 0.2 W/cm^2. A dose of 20 J/cm^2 was delivered on each spot, the treatment time (100 sec) was controlled by a time shutter.

Minipigs. Five animals receiving mTHPC and 5 animals receiving pegylated mTHPC were treated with laser light delivered to the bronchi 12, 24, 48, 72, or 96 hr after drug administration. One pig was assessed for each compound and at each drug-light interval.

For induction, Ketamine, 10 mg/kg body weight, was given i.m. followed by metomidate, 5 mg/kg body weight, and azaperone, 2 mg/kg body weight, both given i.v. Following tracheal intubation, anesthesia was maintained with 0.5 ± 0.05% halothane and 70% nitrous oxide in oxygen. Continuous i.v. infusion of fentanyl 4 μg/kg/h and pancuronium 0.5 mg/kg/h was maintained. The lungs were ventilated with a volume-controlled ventilator with PEEP 3-4 cm H_2O. Tidal volume was maintained at 10 mg/kg and ventilatory frequency adjusted to maintain Pa_{co2} at 4.5–5.5 kPa. A flexible bronchoscope (Olympus) was passed through the endotracheal tube and a suitable area of 0.5 cm diameter was identified at the right upper lobe orifice. A 0.2 mm quartz optical fiber containing a microlens (EPFL Lausanne) was passed through the bronchoscope and argon-pumped dye laser light of 652 nm was delivered to the area to be treated; 20 J/cm^2, 0.2 W/cm^2 were applied as noncontact surface irradiation. The power at the end of the fiber was controlled by a power meter calibrated for 652 nm and the treatment time (100 sec) by a time shutter. Videoassisted equipment allowed for a constant positioning of the lens during treatment with axis of the light beam perpendicular to the area to be treated. After completion of PDT, the animals were extubated. Postoperative pain was controlled using paracetamol i.m.

Assessment of Tissue Damage

Xenografts. Seventy-two hours after light delivery, the mice were euthanised by ether overdose. The whole animals were fixed in neutral-buffered formalin (10%). The tumors and control sites were cut at a right angle to the surface from the center to the periphery, routinely processed, and embedded in paraffin. The extent of necrosis in the irradiated tumors was expressed as an area (measured by planimetry) rather than depth, as this is more accurate for the assessment of inhomogeneous necrosis seen in nodular tumors [14]. A transparent grid with 1 mm spacing was placed over the histological section taken through the largest diameter of the tumor, and the number of grid intersections falling within the necrotic or nonnecrotic tumor area were counted with the aid of a dissecting microscope (magnification ×16). This procedure was repeated three times at different angles, and the median value was used for statistical analysis. Normal tissue damage was assessed by measuring the maximum depth of visible change (necrosis, leucocytic infiltration, edema, and depletion of hair follicles) of the control sites.

Minipigs. At 1, 2, 3, 4, and 7 days after light delivery, the animals were anesthetized and reintubated. Bronchoscopy was performed and the treated areas were inspected and assessed for visible injuries, which were photographed. Seven days after light delivery, the animals were euthanasized after bronchoscopic inspection. Autopsy was performed through a median sternotomy and the treated area was fully excised to establish a geometric profile of tissue destruction. The specimens were fixed in buffered formaline (10%). The irradiated areas were cut at right angles to the

TABLE 1. Extent of Photosensitized Tumor Necrosis for mTHPC[1] and Pegylated mTHPC[2] in Human Squamous Cell Carcinoma and Adenocarcinoma Xenografts (mm^2 ± 1SD)

	Squamous cell carcinoma	Adenocarcinoma
Control	6.5 ± 4.7	11.5 ± 7.6
mTHPC[a]	19.5 ± 3.8	87.6 ± 38.6
Pegylated mTHPC[b]	58.4 ± 23.3	81.5 ± 23.4

[a]0.1 mg/kg mTHPC, light dose 20 J/cm^2, drug-light interval 4 days.
[b]The dose of pegylated mTHPC was equimolar to 0.1 mg/kg mTHPC; light dose 20 J/cm^2, drug-light interval 4 days.

surface through the center to the periphery and were stained with Haematoxylin and Eosin.

Controls. The histological pattern of the tumor xenografts was assessed on three nonsensitized, nonirradiated animals for each type of xenograft to determine the extent of spontaneous tumor necrosis. Untreated bronchi of each minipig were excised well apart from the treated area for control purpose, and the histology was compared to that of the treated area. The extent of tissue injury was assessed by an independent pathologist (HJA) with no prior knowledge of the treatment performed.

Previous studies assessing hyperthermia during PDT have shown that surface irradiance with 20 J/cm^2 and 0.2 W/cm^2 at a wavelength of 652 nm was not associated with temperatures of >38°C at the surface of the treated areas in nude mice bearing xenografts (14).

Statistical analysis. The Student's t-test for unpaired observations was applied where appropriate by using a two-tailed hypothesis. Significance was accepted at $P < 0.05$.

RESULTS

Photosensitization on Xenografts

PDT with 20 J/cm^2 following pretreatment with 0.1 mg/kg mTHPC at a drug-light interval of 4 days resulted in a significantly larger extent of tumor necrosis than in untreated animals ($P < 0.005$) for both tumor types, with larger photosensitized necroses being observed in adenocarcinoma than in squamous cell carcinoma xenografts ($P < 0.001$) (see Table 1). Pegylated mTHPC at an equimolar dose and 20 J/cm^2 at a drug-light interval of 4 days led to a significantly larger extent of tumor necrosis than seen in controls ($P < 0.001$) for both tumor types. Pegylated mTHPC produced a large area of necrosis in both tumor types, but showed only a significant advantage over nonpegylated mTHPC in squamous cell tumors ($P < 0.001$). The control sites revealed leucocytic infiltration in skin and muscle tissues, but no necrosis after PDT with 0.1 mg/kg mTHPC, 20 J/cm^2 and a drug-light interval of 4 days. Photoactivation with an equimolar dose of pegylated mTHPC and the same light dose and drug-light interval did not cause histologically recognizable changes in normal tissue.

Photosensitization on Bronchi of Minipigs

No adverse effects, no atelectasis or pneumonia of the right upper lobe, and no hemoptysis were observed in any animal after PDT using mTHPC or pegylated mTHPC. There was a distinct correlation between the treatment conditions and the PDT-related effects on bronchi: mTHPC caused substantial damage to the bronchial epithelium, underlying glands, and muscle layers of the mucosa, without affecting the bronchial cartilage at drug-light intervals ranging from 12 hr to 2 days. At a drug-light interval of 12 hr, bronchoscopy performed 24 hr after light delivery revealed edema and swelling of the bronchial mucosa and frank ulceration 3 days later (Fig. 1a,b), and histological assessment 7 days after light delivery revealed necrosis of the bronchial epithelium, glands, and muscles of the mucosa with thrombosis of small vessels (Fig. 1c). These alterations became gradually less with increasing drug-light intervals and were no longer observed at an interval of 3 days or more. In contrast, pegylated mTHPC did not result in any visible tissue alteration for any drug-light interval assessed (Fig. 2).

DISCUSSION

PDT has been used investigationally for the treatment of lung cancer since 1980 [19]. Following systemic administration of a photosensitizing agent such as Photofrin, optical delivery systems are engaged to deliver light of a specific wavelength to neoplastic tissue. Centrally located early-stage lung cancer seems to be particularly well suited for PDT, and complete eradication was achieved by PDT alone in superficial tumors <1 cm diameter in 90% of these cases [20]. Preoperative PDT was also applied to centrally located tumors in order to reduce tumor burden and to lessen the extent of pulmonary resection required [21]. Bronchial stump recurrence, however, was not felt by some authors to be a good indication for PDT since tumor recurred in 75% of cases treated

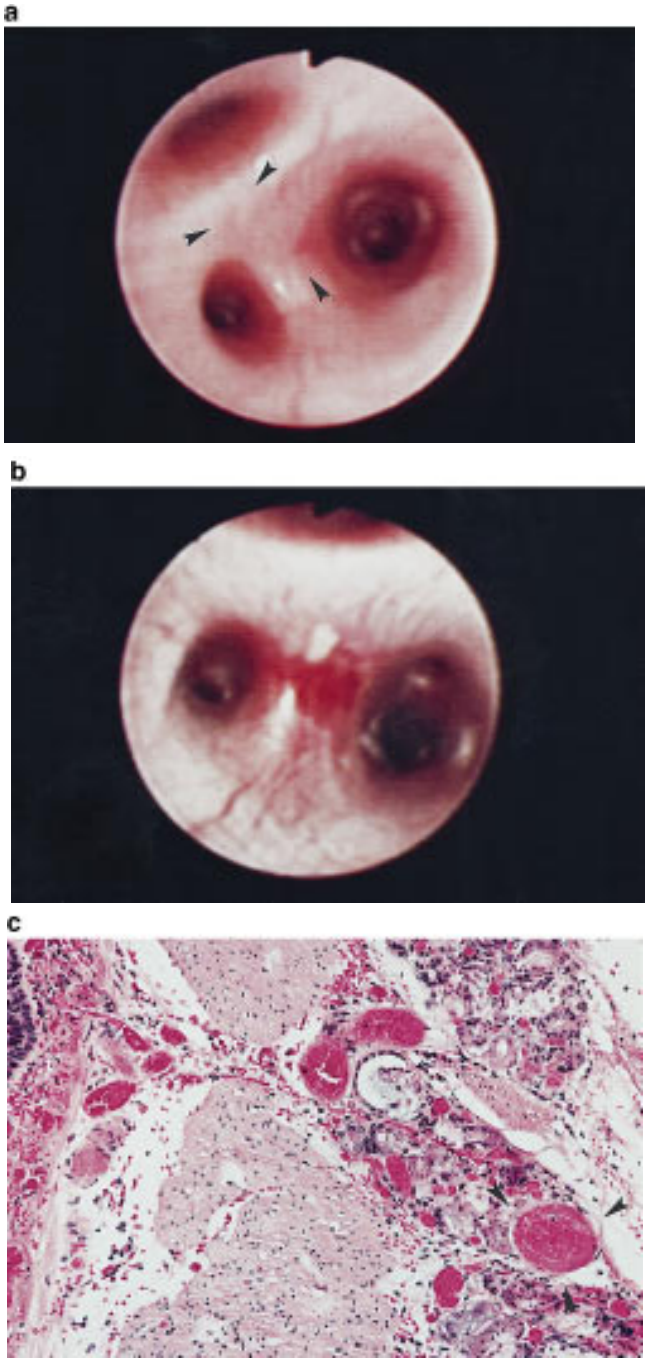

Fig. 1. Photosensitizing effect of 0.1 mg mTHPC/kg and 20 J/cm^2 at a drug-light interval of 12 hr on bronchi of tumor-free minipigs. Bronchoscopy revealed marked swelling of the mucosa (arrows) 1 day **(a),** and a hemorrhagic mucosa with frank ulcerations 4 days **(b),** after light delivery. Histological assessment 7 days after light delivery revealed thrombosis of small vessels (arrows) and necrosis of bronchial epithelium and of glands and muscle layers of the mucosa (H&E, × 100) **(c).**

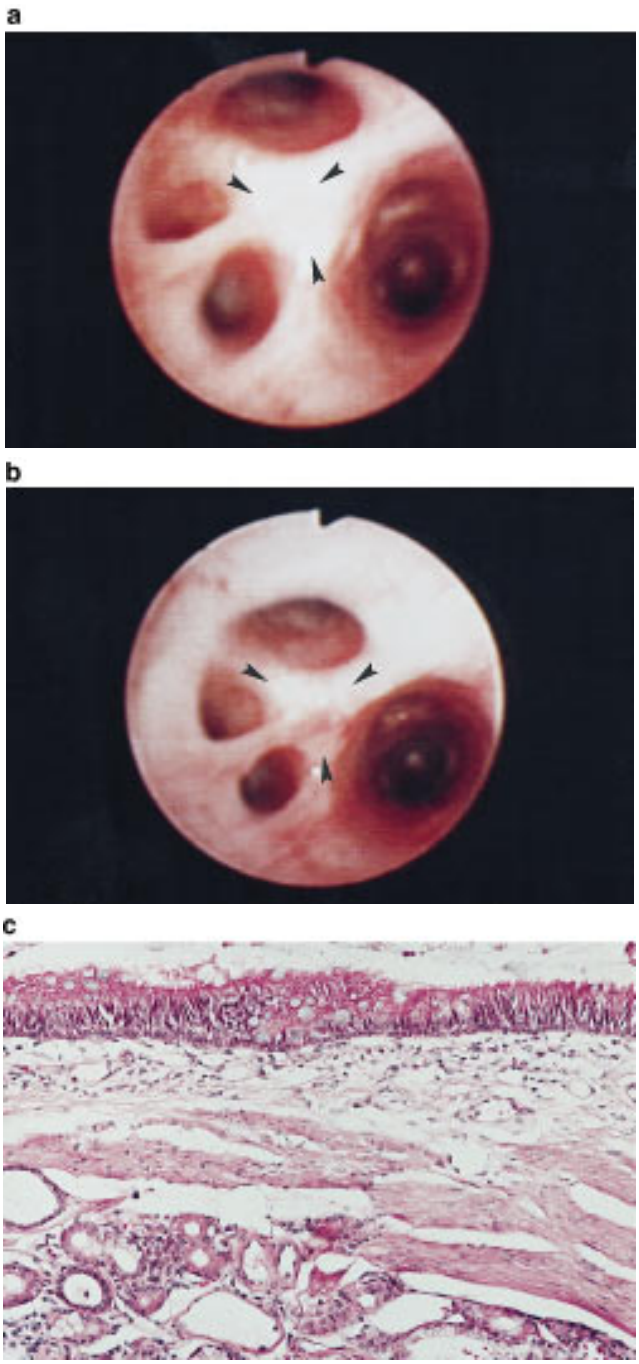

Fig. 2. Photosensitizing effect of polyethylene glycol-derived mTHPC equimolar dosed to 0.1 mg/kg mTHPC and 20 J/cm^2 at a drug-light interval of 12 hr on bronchi of tumor-free minipigs. Bronchoscopy revealed mild edema (arrows), but otherwise no obvious alterations 1 day **(a),** and 4 days **(b),** after light delivery. Histological assessment 7 days after light delivery revealed no visible damage (H&E, × 100) **(c).**

by PDT despite complete response initially [22]. PDT was also investigated for palliation of advanced, inoperable, obstructive bronchial tumors, either alone or in combination with external radiation therapy, stenting, and other recanalisation procedures. PDT resulted in longer-lasting palliation than other procedures such as the Nd:YAG laser [23]. However, side effects after endobronchial PDT were also observed such as long-

lasting skin photosensitization and normal tissue injury [4,24], indicating a need for better tissue selectivity and lesser skin photosensitivity for this purpose.

The effect of PDT depends in a complex way on the characteristics, tissue concentration, and localization of the photosensitizer, on oxygenation and optical properties of the target tissue, and on activation wavelength, power density, and treatment regimen. Efforts to optimize photodynamic therapy were therefore directed toward the development of new photosensitizers [11,25,26], modulations of drug-light conditions [27–30], and improvement of light dosimetry [31]. mTHPC, a chlorin class-derived, second-generation sensitizer has shown promising photosensitizing properties with better therapeutic results than Photofrin in comparative studies and rapid fading of skin photosensitization [28,30,32]. The quantum yield for photoinactivation of cells was smaller for mTHPC than for others sensitizers [32]. Its ability to produce tumor necrosis even with small light doses [12,30] makes mTHPC attractive for clinical trials. mTHPC-PDT was used for various diseases including endobronchial and esophageal tumors with promising results, but normal tissues were also injured [12,28,33–35]. Optimization of mTHPC-PDT is therefore also required.

The first effort to optimize PDT with mTHPC was made by modifying drug-light conditions in an experimental setting with nude mice bearing human malignant xenografts [13,14]. These studies have shown that the efficacy and tumor selectivity of PDT depend strongly on the treatment regimen. A smaller drug dose combined with a higher light dose applied at longer drug-light intervals led to less injury of normal tissues, but extensive necrosis in tumor xenografts in this model [14]. In the present study, 0.1 mg/kg mTHPC and 20 J/cm^2 at an interval of 4 days led to a large extent of photosensitized necrosis in squamous cell carcinoma and adenocarcinoma compared to control animals. However, PDT-related necrosis was significantly larger in adenocarcinoma than in squamous cell carcinoma. In contrast, pegylated mTHPC at an equimolar dose gave a uniform necrosis for both types of xenograft with larger necrosis in squamous cell carcinoma than was observed with mTHPC. This difference in photosensitization between the different xenografts might be attributed to a difference in histology and vascular architecture observed for the different tumour types. Squamous cell carcinoma xenografts were poor in stroma and blood vessels, whereas adenocarcinoma xenografts revealed a delicate tumour stroma with numerous small capillary vessels. Gibson et al. [36] investigated PDT effects on a human and an animal xenograft on the same host and found a different sensitivity of the different tumour types to PDT. They could not explain their findings by a different drug uptake and subcellular distribution of the sensitiser, but rather by a difference in the vascular architecture of the different tumours (36).

In the second part of this experiment, endobronchial mTHPC-PDT was investigated on bronchi of tumor-free minipigs to assess the susceptibility of these structures to tissue injury. The same drug and light dose was applied as in the first part of the experiment. The results suggested that the PDT-related injury on bronchi was strongly related to the treatment conditions. mTHPC at a short drug-light interval led to severe damage of normal bronchi resulting in frank ulceration and necrosis of the bronchial mucosa. These alterations were mainly related to a vascular shutdown with thrombosis of small vessels. The bronchial epithelium and the muscle layers and glands of the mucosa were equally injured, but the cartilage was not affected. These changes became gradually less at longer drug-light intervals and were absent at an interval of 3 days and longer. In contrast, pegylated mTHPC resulted in no visible tissue alterations at any drug-light interval assessed. Our results suggest that photodynamic therapy with pegylated chlorins has the potential to achieve effective antitumor activity while sparing surrounding structures. However, although human malignant xenografts preserve their neoplastic features, they do not reflect the natural growth pattern of endobronchial tumors in patients since these subcutaneously growing tumors do not reveal local invasion. This xenograft model was mainly chosen because of its simplicity and reproducibility, which was required to assess a variety of different drug-light conditions within the same setting. Our results indicated that pegylated mTHPC revealed enhanced photosensitising properties over the nonpegylated compound, which might be related to enhanced targeting of this sensitiser on the tumor, and possibly within a specific site at the tumor cell. Further investigations will be necessary to evaluate this attractive yet complex treatment modality for endobronchial application.

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